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Molecular Characterization and Serology of *Leptospira kirschneri* (Serogroup Grippotyphosa) Isolated from Urine of a Mare Post-Abortion in Brazil

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1 Molecular characterization and serology of *Leptospira kirschneri* (serogroup
2 Grippotyphosa) isolated from urine of a mare post-abortion in Brazil

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13 *France.*

14
15 **Impacts**

- 16
17 • Strains of *L. kirschneri* have apparently never been recovered from horses.
18
19 • Grippotyphosa has been shown to be genetically stable in various hosts and
20 geographical zones.
21
22 • Horses may become unapparent carriers of this organism and shed leptospire in urine.

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31 **SUMMARY**

32 A strain of *Leptospira kirschneri* (serogroup Grippotyphosa) was cultured from
33 urine of a mare post-abortion in Brazil, and characterized by serogrouping, MLVA,
34 PGFE, and sequencing of genes *rrs* and *secY*. Strains of *L. kirschneri* have apparently
35 never been recovered from horses in tropical area, only in Europe and USA. Knowledge
36 of local epidemiology is important to interpret genetic profiles of leptospires circulating
37 in an area.

38

39 **Keywords:** *Leptospira kirschneri*; Grippotyphosa; Horse

40

41

42 **Introduction**

43 Leptospirosis is an important infectious disease in livestock caused by spirochetes
44 belonging to the genus *Leptospira*, which is reported worldwide, particularly in tropical
45 countries (Martins and Lilenbaum, 2013). Infected animals (as cattle, sheep, goats, pigs
46 and horses) often present a chronic form, with impaired fertility, abortion, stillbirth, and
47 decreased milk production. Although most reported cases of urban leptospirosis in
48 humans in Brazil are caused by *Leptospira interrogans*, particularly serovar
49 Copenhageni (Silva et al., 2009), infection in livestock seems to be majorly determined
50 by other serogroups, as Sejroe for ruminants and Australis for horses (Martins and
51 Lilenbaum, 2013). Leptospirosis regarded as a zoonosis, humans become infected
52 through either direct contact with the urine or other biological materials from the
53 infected animals or indirect contact with water, soil and vegetation polluted with urine
54 from animals harbouring pathogenic leptospires (Foronda et al. 2009).

55 Leptospirosis is considered a neglected zoonosis. Since leptospirosis
56 transmission to humans can occur through contact with urine of animal reservoirs or
57 exposure to an environment contaminated with leptospires, the contemporary concept of
58 "One Health" is particularly appropriate for these organisms, due to its epidemiology,
59 especially in tropical countries (Chappel and Smythe, 2012). In that regard, increases
60 and expansion of human populations, including encroachment on wildlife habitat,
61 increase opportunities for animal-human interactions. Various biomes and ecosystems
62 promote exposure to various *Leptospira* strains, as rural and urban environments.
63 Furthermore, global climate change is also apparently promoting the spread of
64 leptospirosis. In that regard, increased temperatures may enhance survival of leptospires
65 in neofomed environments and may result in an expansion of the habitats occupied by
66 animal reservoirs of the bacterium (Lau et al. 2010).

67 Leptospiral infection can be host-maintained, if transmitted readily among
68 members of host species, or incidental when such transmission does not normally occur.
69 Incidental infections are usually more severe than those in maintenance hosts (Chappel
70 and Smythe, 2012). Detecting carrier animals is vital to understanding enzootic and
71 epizootic leptospirosis in a particular environment (Foronda et al., 2009). Generally the
72 incidence in various hosts as well as the infecting serovars varies considerably among
73 geographical regions (Arent et al., 2013; Chappel and Smythe, 2012; Verma et al.,
74 2013).

75 Horses may become unapparent carriers and shed leptospire [in](#) urine, thereby
76 serving as reservoirs and sources of infection for other animals, including humans
77 (Hamond et al., 2013). The reproductive syndrome of equine leptospirosis was recently
78 reviewed; the most common serogroup recovered from equine abortions was Pomona,
79 whereas other serogroups (Australis, Icterohaemorrhagiae, Sejroe) have also been
80 isolated from aborted equine fetuses in several countries (Verma et al., 2013; Hamond
81 et al., 2014). However, *Leptospira kirschneri* serogroup Grippytyphosa has apparently
82 never been recovered from horses in [tropical area](#).

83 Therefore, the purpose of this study was to describe recovery and characterization of
84 *Leptospira kirschneri* (serogroup Grippytyphosa) from urine of a mare post-abortion
85 (and the aborted fetus), as well as serological findings in this mare and her herd mates.

86

87 **Methods**

88 **Study Design**

89 Twelve mares (aged 7-12 y) from the same herd (extensive breeding) in the state
90 of Rio de Janeiro, Brazil, were studied. These mares had a history of reproductive
91 problems (mainly abortions) and had not been vaccinated [and](#) nor treated for

92 leptospirosis. Blood samples for serology were collected (jugular venipuncture) into
93 evacuated tubes (Vacutainer[®], BD Diagnostics, Franklin Lakes, NJ, USA).
94 Additionally, urine samples were collected by probing (Human nasogastric probe n° 18
95 – Embramed, São Paulo, SP, Brazil) and put into 50 mL sterile vials (BD, Franklin
96 Lakes, USA) and immediately inoculated into 5 mL culture media tubes (EMJH). A 2
97 mL aliquot was chilled and transported to the laboratory for PCR. During the study, one
98 mare (age 8 y), originating from Europe and living in Brazil for the last 2 y, aborted
99 (seventh month of pregnancy). The fetus was necropsied on the following day; it had
100 jaundice and widespread petechial haemorrhages. Samples of kidney and liver were
101 collected for culture and PCR.

102 **Serology**

103 For detection of anti-*Leptospira* antibodies, a microscopic agglutination test
104 (MAT) was used, with a complete panel (28 serovars representing 24 serogroups;
105 Institute Pasteur - Paris, France), according to international standards (World
106 Organization for Animal Health, 2012). The serogroup (serovar) with the highest titre
107 was regarded as infective. Samples were considered reactive when for titers ≥ 200 , and
108 whereas titres ≥ 800 were considered strongly reactive and indicative of an acute
109 infection (Martins and Lilenbaum, 2013).

110

111 **Bacteriological Culture**

112 A few drops of urine from each of the 12 mares and the fetal kidney and liver were
113 immediately inoculated into tubes containing 5 mL of EMJH liquid media (Difco
114 Laboratories, Franklin Lakes, NJ, USA) and 5 mL semisolid Fletcher media (Difco
115 Laboratories). At the laboratory, tubes were incubated at 28 °C and examined under
116 darkfield microscopy once weekly for 20 wk (Faine et al., 2000).

117 **PCR protocol**

118 All DNA samples (urine from the 12 mares and liver/kidney from the aborted
119 foal) were extracted using the Promega Wizard SV Genomic DNA Purification System®
120 (Promega, Madison, WI, USA). Primers used were targeted to the *lipL32* gene
121 (regarded as present only in pathogenic leptospires) as described (LipL32_45F - 5'AAG
122 CAT TAC TTG CGC TGG TG 3' and LipL32_286R - 5'TTT CAG CCA GAA CTC
123 CGA TT 3'), which generate a 242 bp fragment (Stoddard et al. 2009). Briefly, primers
124 were used in a concentration of 0.6 µM, 1.0 U Taq polymerase, 2.4 µM MgCl₂, and 0.3
125 mM dNTP in a final volume of 25 µL. One cycle of initial denaturation at 94 °C for 2
126 min, was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing the primers
127 to 53 °C for 30 s, 1 min extension at 72 °C, and a final extension cycle at 72 °C for 5
128 min. Strain *Leptospira interrogans* serovar Copenhageni, Fiocruz L1-130 (ATCC BAA-
129 1198) was used as a positive control. To minimize false-negative results, an internal
130 DNA control was designed and synthesized (IDT – Integrated DNA Technologies,
131 Coralville, IA, USA). The synthetic gene had a 121 bp portion of the *lipL32* gene in
132 each extremity, whereas in the middle has a gene part of the sequence *ligB*, yielding a
133 total DNA sequence of 754 bp (Hamond et al., 2014).

134

135 **Characterization of the isolate**

136 The isolate was serogrouped using a panel of 32 specific antisera provided by the
137 Royal Tropical Institute (KIT, Amsterdam; Faine et al., 2000). Furthermore, its DNA
138 was extracted and a partial sequence of the *rrs* (Merien et al., 1993) and *secY* genes
139 (Ahmed et al., 2006) were amplified by PCR and sequenced. The latter procedure was
140 done at the Genotyping of Pathogens and Public Health Platform (*Institut Pasteur*,
141 Paris, France). All molecular epidemiological data were stored and analyzed with

142 Bionumerics software (Version 6.5; Applied-Maths, Sint-Martens-Latem,
143 Belgium). Genotyping was also performed by multiple-locus variable-number tandem
144 repeat analysis (MLVA) using the loci VNTR4, VNTR7, and VNTR10, as described
145 (Salaun et al., 2003). According to the analysis of partial sequencing of the gene *secY*,
146 PFGE was conducted using *Not* I restriction enzyme (Herrmann et al., 1992), to
147 compare DNA of the isolate to that of other strains of the same species and serogroup.

148

149 **Results**

150 Four of the 12 (33%) tested sera were reactive, all of them against serogroup
151 Grippotyphosa (sv. Grippotyphosa). The mare that had aborted had a titre of 400,
152 whereas the three others had titres of 200.

153 In this study, PCR detected leptospiral DNA in the urine of three of 12 (25%)
154 mares, of which two were seroreactive, although the remaining mare was seronegative.
155 From those mares, only the urine of the mare that had aborted yielded a pure culture of
156 leptospire.

157 Based on serogrouping, the isolate belonged to Grippotyphosa serogroup (titre
158 12,800), whereas sequencing products of *rrs* and *secY* partial genes characterized it as
159 *Leptospira kirschneri* genomospecies. Furthermore, based on *secY* nucleotide sequences
160 (Fig. 1), it was similar to sv Grippotyphosa strains Moskva V (isolated from humans in
161 Russia) and 200901480_Mayotte (from humans in Mayotte), as well as to sv
162 Vanderhoedeni strain Kipod 179 (from a hedgehog in Israel).

163 Furthermore, MLVA-VNTR 4 (497 bp), VNTR 7 (372 bp) and VNTR 10 (830
164 bp) analysis also confirmed that our isolate was sv Grippotyphosa (Fig. 2), with a
165 profile closely related to the reference strain Moskva V. Finally, PFGE of the isolate

166 with other *L. kirschneri*/Grippotyphosa strains, such as Moskva V and Grippotyphosa
167 strain 200901480, showed a very close profile among the three strains (Fig 3).

168

169 **Discussion**

170 The occurrence of anti-*Leptospira* agglutinins for serogroup Grippotyphosa was
171 unexpected, since horses from the same region are typically seroreactive for serogroups
172 Icterohaemorrhagiae or Australis (Hamond et al. 2013), as are many humans in Brazil
173 (Silva et al., 2009).

174 It is not clear if the mare that had aborted has acquired the infection in Brazil or
175 if it was a chronic infection acquired in Europe, at least 2 y before the abortion
176 occurred. [Nevertheless, that mare](#) had already delivered a healthy foal in the past year,
177 and that other mares from the same herd also seroreacted against that serovar, we
178 inferred it was more likely to be a locally acquired infection. Although common in
179 Europe (Arent et al., 2013), Grippotyphosa serogroup is not the most prevalent in
180 tropical regions, where members of Icterohaemorrhagiae serogroup seem to be
181 predominant, as well as serogroup Australis (sv Bratislava), regarded as adapted to
182 horses (Hamond et al., 2014). Grippotyphosa is usually associated with environmental
183 contamination, and is maintained by various wildlife species (de Carvalho et al., 2014).
184 There are reports regarding isolation of that serogroup from animals, including
185 abortions in sheep in Canada (Kingscote, 1985), cattle in the USA (Hanson et al., 1964),
186 [horse with uveitis in Europe \(Hartskeerl et al., 2004\) and abortion in US \(Erol et al.,](#)
187 [2015\).](#)

188 Although bacteria cultures were negative, PCR detected leptospiral DNA from
189 the kidney of the aborted foal, thereby confirming the cause of the abortion.

190 Furthermore, *L. kirschneri* was detected by PCR in the tissues of a premature foal

191 (Vemulapalli et al., 2005), but recovery of that species in pure culture, as well as
192 molecular characterization of the isolate, has apparently never been reported in horses in
193 tropical area.

194 Sequencing of *secY* in DNA extracted from the clinical isolates samples allowed
195 a simple and rapid first-line screening and identification of the presumptive serovar. It
196 has already been conducted on isolates from human origin (Bourhy et al., 2013).

197 Although analysis based on a phylogenetic tree of *secY* fragments is very useful for
198 identifying the presumptive serovar, it has not commonly been performed on isolates
199 from animal origin and therefore, should be encouraged in future studies.

200 The MLVA analysis is a simple and rapid PCR-based method for identification
201 of most serovars of *L. interrogans* and *L. kirschneri* (Bourhy et al. 2013; Zilber et al.
202 2014). Therefore, its use should also be encouraged for a fast and simple DNA-based
203 characterization of leptospiral isolates.

204 The PFGE analysis of *Not* I-digested genomic DNA revealed a very close profile
205 among the three strains, as Moskva V and Grippytyphosa strain 200901480, which is
206 remarkable, given the distances (South America, Russia and Indian Ocean) and distinct
207 hosts (man and horse). Notwithstanding, that finding reinforces the One Health concept
208 regarding human/animal leptospirosis, which should be considered in future approaches
209 regarding the diagnosis and control of leptospirosis worldwide.

210 In conclusion, this was apparently the first report of *L. kirschneri* to have been
211 isolated from a horse in tropical area. In addition, it is noteworthy that characterizing the
212 genetic profile of the leptospirosis strains circulating in an area is very important to
213 interpret local epidemiology of this organism.

214

215

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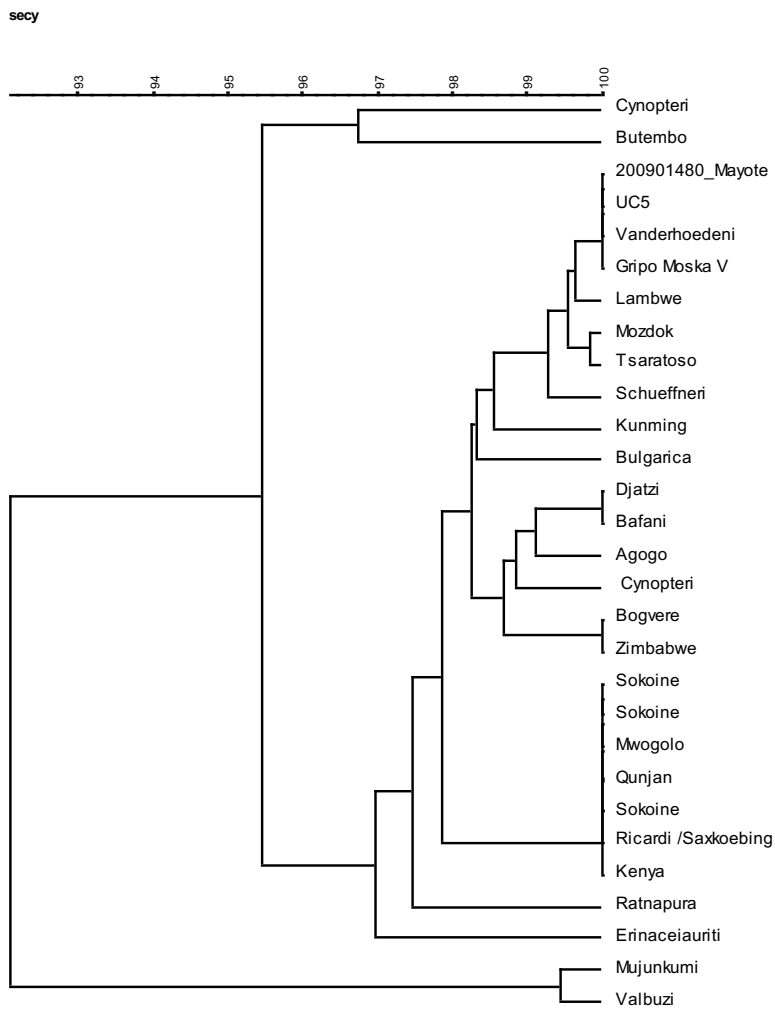
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- 312

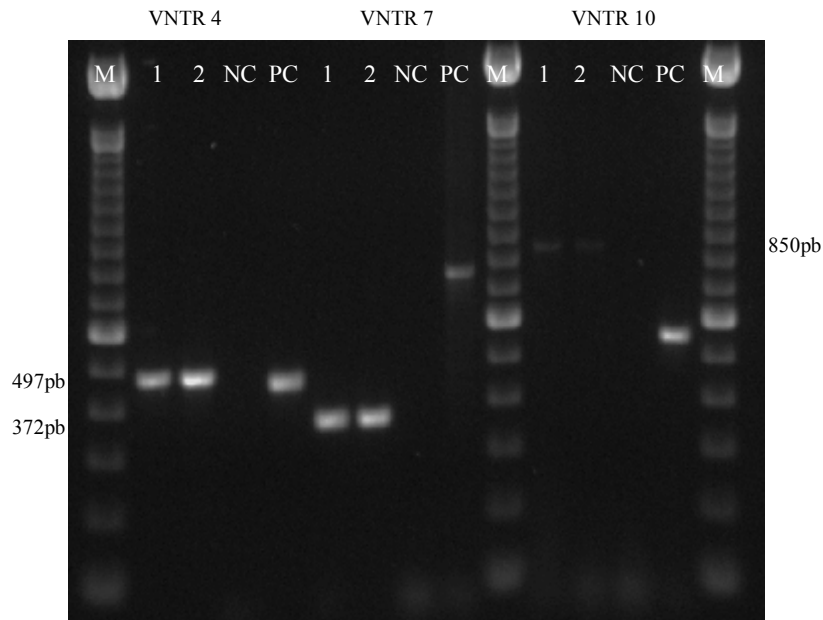
313 Fig 1. Phylogenetic tree of leptospiral *secY* partial gene sequences of reference strains
314 of *L. kirschneri* species, including the isolate obtained from a mare post-abortion (UC5).



315

316

317 Fig 2. PCR analysis of the polymorphism of two representative VNTR loci.
318 Amplification was performed on the VNTR4, VNTR7 and VNTR10 loci of *L.*
319 *kirschneri* sv Grippotyphosa Moskva V and isolate UC5 indicates *Leptospira* serovars



M- 100pb DNA ladder 1-UC5; 2-*L.kirschneri* sv Grippotyphosa Moskva V; NC- Negative Control; PC – Positive control (*L.interrogans* sv Canicola)

320

321

322 Fig 3. PFGE (using *Not* I restriction enzyme) from the recovered sample (UC5)
323 compared to other *Leptospira kirchneri* serogroup Grippotyphosa, as Moskva V and
324 strain 200901480.

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